Exhibit D

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SHORT COMMUNICATION

TH1 and TH2 cytokine production by peripheral blood mononuclear cells from HIV-infected patients

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Objective: To study the TH1–>TH2 cytokine switch, thought to occur during the progression of HIV infection.

Design: We investigated interleukin (IL)-2, interferon (IFN)-γ, IL-4, IL-6 and IL-10 production by phytohaemagglutinin (PHA)-stimulated and unstimulated peripheral blood mononuclear cell (PBMC) cultures from HIV-negative controls and HIV-positive subjects, stratified according to the Centers for Disease Control and Prevention (CDC) criteria. We correlated the above parameters with markers of disease progression.

Methods: Cytokine production was measured in supernatants using enzyme-linked immunosorbent assay (ELISA) in 40 patients and 17 controls. To evaluate disease progression, we also determined CD4+ cell counts, PHA-induced proliferative response, p24 release and spontaneous immunoglobulin (Ig) G and IgM production.

Results: In agreement with the TH1–>TH2 switch hypothesis, we found that in the course of HIV disease mitogen-stimulated IL-2 production decreased, spontaneous and stimulated IL-6 production and spontaneous IL-10 secretion increased; IL-4 showed an increasing trend, although it was reduced in HIV-positive subjects. Finally, immunoglobulin production increased over time. In contrast, mitogen-stimulated IFN-γ and IL-10 production did not change among the CDC categories, although the former decreased and the latter increased in comparison with HIV-negative controls.

Conclusions: Our data partially agree with the TH1–>TH2 switch hypothesis. Since IL-6 and IL-10 are produced by different cell types, whose proportions and functional features vary in the course of the disease, further experiments with purified lymphocyte subsets and monocytes are required. Nevertheless, as already suggested, we believe that a switch from a type 1 to a type 2 response occurs in HIV infection.

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Introduction

Loss of T-helper (TH) function occurs long before the depletion of CD4+ cells, suggesting that factors other than CD4 depletion also contribute to HIV-associated immunodeficiency [1–5]. There has been increased interest in the role of immunoregulatory cytokines [5,6], hormones [7,8] and neuropeptides

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[9]. Recently, it has been suggested that a switch from a TH1-like to a TH2-like cytokine pattern occurs during the progression of HIV infection, and that it may contribute both to disease progression and to increased susceptibility to opportunistic infections and malignancies [4–6].

However, several findings appear to contradict this hypothesis. First, cytokine gene expression in lymph nodes from HIV-infected subjects showed no decrease in interleukin (IL)-2 and interferon (IFN)-γ and an increase in IL-4 and IL-10, but little IL-4 at all stages of the disease; in addition, macrophages, but not T cells, displayed increased IL-10 mRNA levels at all stages [10]. Second, elevated levels of IFN-γ mRNA and lowered IL-2 mRNA were observed in HIV-positive peripheral blood mononuclear cell (PBMC) cultures, while IL-4 and IL-10 mRNA were not significantly changed. Furthermore, changes in cytokine gene expression were markedly different in CD4 and CD8 T cells, since IL-10 and IFN-γ but not IL-2 were increased compared with HIV-negative controls only in the former [11]. Finally, increased IL-4 or some sort of TH2 switch was not confirmed in about 100 HIV-positive TH clones [10].

In this study we investigated IL-2, IFN-γ, IL-4, IL-6 and IL-10 production by mitogen-stimulated and unstimulated PBMC cultures from HIV-positive subjects, stratified according to the Centers for Disease Control and Prevention (CDC) criteria, and we correlated the above parameters with markers of disease progression.

Methods

Subjects

We studied 40 HIV-positive subjects, including 11 women and 29 men (mean age, 37.2±7.1 years; range, 23–55 years), classified according to CDC criteria [12] on the basis of CD4+ cell counts: 500×10^6/l (category 1; n = 7), 200–499×10^6/l (category 2; n = 12) and $<200\times10^6$/l (category 3; n = 21). The distribution of risk categories [12 ex-intravenous drug users (IVDU), 17 homosexuals and 11 heterosexuals] was comparable in the three CDC groups. We included 17 healthy HIV-negative controls matched for age and sex. Zidovudine was administered to 24 of the 40 at a daily dosage of between 500 and 750 mg.

Lymphocyte separation and culture conditions

PBMC, separated from heparinized blood by gradient centrifugation according to Boyum [13], were resuspended in RPMI-1640 culture medium plus 10% heat-inactivated fetal calf serum (FCS; Gibco, Grand Island, New York, USA), 1% glutamine and antibiotics. Macrocultures were set up with 2×10^6/ml PBMC in 1 ml final volume in the presence of phytohaemagglutinin (PHA) (Gibco) 4 mg/ml or medium alone. After 48 h, culture supernatants recovered by centrifugation were tested for cytokine and p24 content [14]. Microcultures for the evaluation of mitotic responses were set up as described previously [15]. p24 antigenaemia in culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA; HIVAg-1 monoclonal; Abbott Diagnostic Division, Illinois, USA) according to the manufacturer's specifications. Spontaneous polyclonal immunoglobulin (Ig) G and IgM production was evaluated by ELISA [16], although the culture period was not the optimal standardized previously [17].

Measurement of cytokines

The following cytokines were evaluated: (1) IL-2 by competitive radioimmunoassay (Medgenix, Fleurus, Belgium); (2) IFN-γ by immunoradiometric assay (IRMA, Medgenix); (3) IL-4 and IL-6 by ELISA assay (Research and Diagnostics Systems, Minneapolis, Minnesota, USA); and (4) IL-10 by competitive enzyme immunoassay (Assay Research Inc., College Park, Maryland, USA). All assays were performed according to the manufacturers' specifications.

Statistical analysis

Results were analysed using non-parametric tests (Wilcoxon's signed-rank test and Mann–Whitney's test). Correlation coefficients were calculated using Spearman's test. A two-tailed P value < 0.05 was considered significant.

Results

PHA-stimulated IL-2, IL-4 and IFN-γ, particularly IL-2, were significantly reduced in HIV-positive patients (Figs 1 and 2), whereas unstimulated cytokine production was comparable in HIV-positive and HIV-negative subjects. Considering disease progression, IL-2 decreased and IL-4 increased; in addition, the former correlated positively with absolute CD4+ cell counts ($r = 0.294$; $P = 0.033$, Spearman correlation coefficient) and the latter with both PHA-stimulated and unstimulated p24 release ($r = 0.38$; $P = 0.008$ and $r = 0.49$; $P = 0.005$, respectively). IL-2/IL-4 ratio was significantly reduced in HIV-positive versus HIV-negative subjects ($P = 0.0001$, Mann–Whitney test) and decreased during the course of disease; in fact, the ratio was significantly reduced in category 3 compared with category 1 and negatively correlated with p24 release ($r = -0.5037$; $P < 0.001$). Mitogen-induced IFN-γ production did not show a clear relationship with disease progression, although it was negatively correlated with PHA-stimulated p24 release ($r = -0.274$;

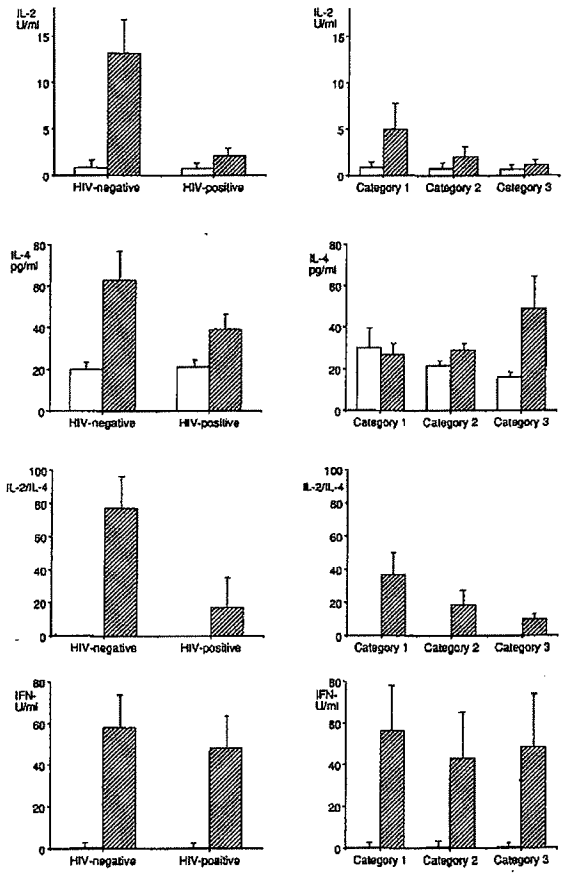

Fig. 1. Spontaneous (□) and phytohaemagglutinin (PHA) at 4 μg/ml-stimulated (▨) interleukin (IL)-2, IL-4, interferon (IFN)-γ production and mitogen-induced IL-2/IL-4 ratio in all HIV-positive and HIV-negative subjects (left panel) and in HIV-positive individuals stratified according to the Centers for Disease Control and Prevention (CDC) categories (1 = CD4 500×10^6/l, 2 = CD4 $200–499 \times 10^6$/l, 3 = CD4 $< 200 \times 10^6$/l; right panel). Values are the mean ± SE. Statistically significant comparisons (Mann–Whitney test): PHA-stimulated IL-2 production: HIV-negative versus HIV-positive individuals, $P = 0.001$; category 2 versus HIV-negative subjects, $P = 0.0001$; category 3 versus HIV-negative subjects, $P < 0.0001$. PHA-stimulated IFN-γ production: HIV-negative versus HIV-positive individuals, $P = 0.005$; category 2 versus HIV-negative subjects, $P = 0.037$; category 3 versus HIV-negative subjects $P = 0.0023$. PHA-stimulated IL-4 production: HIV-negative versus HIV-positive individuals $P = 0.012$; category 1 versus HIV-negative subjects, $P = 0.033$; category 2 versus HIV-negative subjects, $P = 0.039$; category 3 versus HIV-negative subjects, $P = 0.0506$. IL-2/IL-4 ratio: HIV-negative versus HIV-positive individuals, $P = 0.0001$; category 2 versus HIV-negative subjects, $P = 0.0024$; category 3 versus HIV-negative subjects, $P = 0.0001$; category 1 versus category 3, $P = 0.0086$.

$P = 0.043$) and positively with PHA-induced IL-2 production ($r = 0.393$; $P = 0.006$). Spontaneous IL-6 secretion was significantly greater in HIV-positive subjects, whereas mitogen-induced production was not. Both spontaneous and PHA-stimulated IL-6 production increased with disease progression, and the

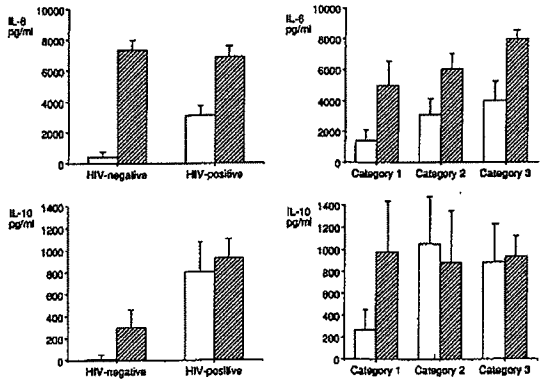

Fig. 2. Spontaneous (□) and phytohaemagglutinin (PHA) at 4 μg/ml-stimulated (▨) interleukin (IL)-6 and IL-10 production in all HIV-positive and HIV-negative subjects (left panel) and in HIV-positive individuals stratified according to the Centers for Disease Control and Prevention (CDC) categories (1 = CD4 500×10^6/l, 2 = CD4 $200–499 \times 10^6$/l, 3 = CD4 $< 200 \times 10^6$/l; right panel). Values are the mean ± SE. Statistically significant comparisons (Mann–Whitney test): unstimulated IL-6: HIV-negative versus HIV-positive individuals, $P = 0.001$; category 2 versus HIV-negative subjects, $P = 0.0027$; category 3 versus HIV-negative subjects, $P = 0.01$. Unstimulated IL-10: HIV-negative versus HIV-positive individuals, $P = 0.029$; category 3 versus HIV-negative subjects, $P = 0.023$.

former negatively correlated with CD4+ cell counts and PHA-induced IL-2 secretion ($r = -0.311$; $P = 0.025$ and $r = -0.342$; $P = 0.04$, respectively). PHA-stimulated and unstimulated IL-10 production were higher in HIV-positive subjects, although only the latter significantly. Spontaneous cytokine secretion was low in category 1 and increased in categories 2 and 3 up to values observed in mitogen-stimulated cultures; in contrast, PHA-stimulated IL-10 production was comparable in all the CDC categories. Both spontaneous and PHA-stimulated IL-10 production positively correlated with PHA-induced IL-4 production ($r = 0.407$; $P = 0.017$ and $r = 0.267$; $P = 0.048$, respectively), and mitogen-induced IL-10 production positively correlated with PHA-stimulated and spontaneous IL-6 production ($r = 0.384$; $P = 0.007$ and $r = 0.415$; $P = 0.016$, respectively). Spontaneous IgG and IgM secretion was greater in HIV-positive subjects and increased with disease progression; it correlated negatively with PHA-induced IL-2 (IgM: $r = -0.33$; $P = 0.046$; IgG: $r = -0.533$; $P = 0.002$) and IFN-γ production (IgG: $r = -0.355$; $P = 0.032$) and correlated positively with spontaneous IL-6 production (IgG: $r = 0.428$; $P = 0.013$). Spontaneous and mitogen-induced p24 release was present in HIV-positive subjects and increased with disease progression (Fig. 3).

Proliferative responses to PHA were significantly reduced in all the CDC categories compared with HIV-negative subjects, although a definite relationship with disease progression was not found (data not shown). In HIV-positive subjects, the prolifera-

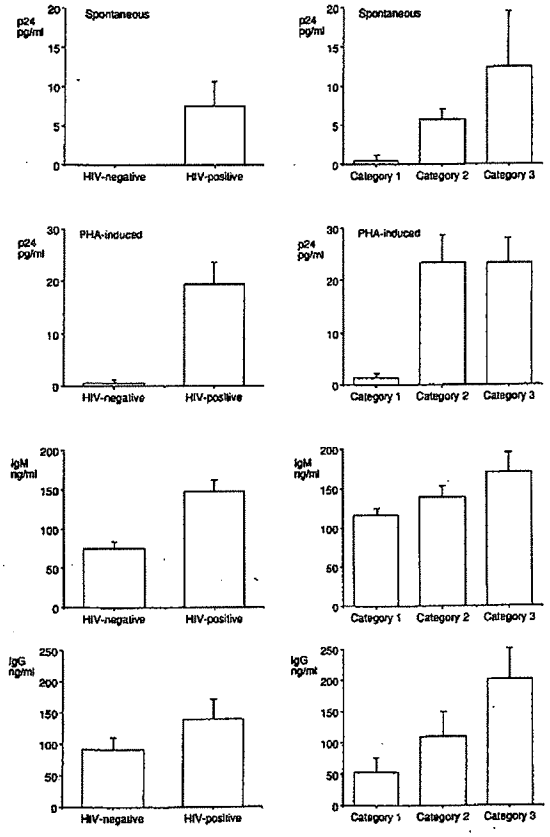

Fig. 3. Spontaneous and phytohaemagglutinin (PHA) at 4 μg/ml-stimulated p24 release and spontaneous immunoglobulin (Ig) M and IgG production in all HIV-positive and HIV-negative subjects (left panel) and in HIV-positive individuals stratified according to the Centers for Disease Control and Prevention (CDC) categories (1 = CD4 500×10^6/l, 2 = CD4 < 200–499×10^6/l, 3 = CD4 < 200×10^6/l; right panel). Values are the mean ± SE. Mean values ± SE of CD4+ cell counts in the three CDC categories were 657 ± 48, 330 ± 24 and 84 ± 16×10^6/l, respectively. Statistically significant comparisons (Mann–Whitney test): PHA-stimulated p24: HIV-negative versus HIV-positive individuals, $P = 0.001$; category 2 versus HIV-negative subjects $P < 0.0001$; category 3 versus HIV-negative subjects, $P < 0.0001$; category 1 versus category 2, $P = 0.005$; category 1 versus category 3, $P = 0.006$. Spontaneous p24: HIV-negative versus HIV-positive individuals $P = 0.001$; category 1 versus HIV-negative subjects, $P = 0.023$; category 2 versus HIV-negative subjects, $P < 0.0001$; category 3 versus HIV-negative subjects, $P < 0.0001$; category 1 versus category 2, $P = 0.007$; category 1 versus category 3, $P = 0.008$. Spontaneous IgM: HIV-negative versus HIV-positive individuals, $P < 0.0001$; category 1 versus category 3, $P = 0.0087$. Spontaneous IgG: category 1 versus category 3, $P = 0.0437$. Immunoglobulin secretion in HIV-positive subjects correlated negatively with CD4+ cell counts (IgM: $r = -0.465$, $P = 0.007$; IgG: $r = -0.483$, $P = 0.005$), and spontaneous IgM secretion correlated positively with spontaneous and stimulated p24 release ($r = 0.519$, $P = 0.003$ and $r = 0.445$; $P = 0.01$, respectively).

tive response correlated positively with stimulated IL-6 and with spontaneous IL-6 and IL-10 production, whereas in HIV-negative subjects blastogenesis correlated negatively with mitogen-stimulated and spontaneous IL-10 production and correlated positively with mitogen-induced IL-2 production (data not shown). Finally, we did not observe differences among cytokine profiles adjusting for sex, risk factors and zidovudine treatment.

Discussion

The data presented here partially agree with the TH1–>TH2 switch hypothesis. We found that IL-2 was reduced in HIV-positive subjects and decreased from category 1 to category 3; PHA-stimulated IL-4 production showed an increasing trend during disease progression and correlated positively with p24 release. Moreover, the IL-2/IL-4 ratio decreased with disease progression, being significantly reduced in category 3 versus category 1, and correlated negatively with p24 release. However, in disagreement with the TH1–>TH2 theory, IL-4 was decreased in the HIV-positive subjects, in accordance with Re *et al.* [18], but in contrast with Clerici and Shearer [5], and Fan *et al.* [11]. Furthermore, IFN-γ, even if slightly reduced in HIV-positive subjects and negatively correlating with p24 release, did not change among the CDC categories, in contrast with Fan *et al.* who found augmented IFN-γ gene expression in PBMC from HIV-infected individuals [11]. In agreement with the literature [19–21], PHA-stimulated and spontaneous IL-6 production was increased in HIV-positive subjects. Moreover, IL-6 increased with disease progression, correlated negatively with IL-2 production and positively with IL-10. Since IL-6 may up-regulate viral replication in lymph nodes enhancing the viral burden and thus the progress from clinical latency to overt AIDS [20,21], our data are in line with the role of IL-6 in disease progression and with the TH1–>TH2 switch hypothesis. Moreover, IL-6, which is spontaneously secreted by monocytes and B lymphocytes in HIV infection [19,21], might contribute to polyclonal B-cell activation and to the development of HIV-associated lymphomas [22]. We found that spontaneous immunoglobulin production was greater in HIV-positive subjects, increased with disease progression, and correlated positively with IL-6 and negatively with IL-2 and IFN-γ production.

Immunoglobulin synthesis, as well as IL-2/IL-4 ratio, was significantly different in categories 1 and 3. Moreover, category 1 and HIV-negative subjects did not differ with regard to IL-2, IFN-γ and IL-6 production, whereas categories 2 and 3 did. These data suggest a TH1 profile in category 1, in contrast to advanced-stage patients, further supporting the TH1–>TH2 switch hypothesis.

IL-10 has powerful immunosuppressive activities, inhibiting antigen-presenting cell function, cytokine

synthesis by macrophages and antigen specific T-cell proliferation via monocytes [23–25]. Conversely, it enhances both proliferation and immunoglobulin production by B lymphocytes [26], and is constitutively secreted by B-cell lines derived from patients with AIDS and Burkitt's lymphoma, thus contributing to the polyclonal B-cell activation and hypergammaglobulinaemia seen in these patients [27]. Finally, IL-10 inhibits TH1 cytokine synthesis [23], thus modulating the balance between TH1 and TH2 responses. In accordance with the proposed TH1–>TH2 switch hypothesis, we found that IL-10 production was increased in HIV-positive subjects, and correlated positively with IL-4 and IL-6, and that spontaneous IL-10 secretion abruptly increased in category 2, closely resembling the behaviour of p24 release. However, mitogen-stimulated IL-10 production was comparable in all CDC categories, and showed no correlation with TH1 cytokines, spontaneous Ig synthesis and parameters of disease progression. It is noteworthy that IL-10 is produced by a wide variety of cells, including macrophages, B and T lymphocytes [23]. IL-10 is produced mostly by macrophages in lymph nodes from HIV-positive subjects [10], but by CD4+ T cells in peripheral blood [11]. Since CD4+ cells, monocytes and B lymphocytes are present in varying proportions at different stages of disease, we cannot exclude the possibility that the different cytokine profiles observed might be related to variations in the cell composition of unfractionated PBMC cultures. This could be the *in vitro* equivalent of the *in vivo* dropping out or anergy of CD4 cells, which simply gives the impression of a switch from TH1 to TH2 cytokine responses [10].

These findings reinforce the need for further experiments with purified cells from peripheral blood or lymph nodes to determine the types of cells responsible for increased IL-10 production and thus its precise role in the natural history of the disease. Whether a TH1–>TH2 switch occurs and is in some way responsible for disease progression is still undetermined. Nevertheless, our data agree with Clerici and Shearer [5], who are confident that a switch from a type 1 to a type 2 response occurs in HIV infection.

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